

Antidepressant-like and neuroprotective effect of hydroalcoholic extract of *Aloysia citriodora* in animals subjected to childhood stress

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Abstract

Stress in childhood can harm brain development, leading to biological and systemic dysfunctions that, along with genetic factors, may contribute to major depressive disorder (MDD) in adulthood. MDD treatment is complex, with current drugs taking time to show effects and not working for many patients. As a result, medicinal plants, like *Aloysia citriodora* (*A. citriodora*), are being studied as treatments for resistant depression. The main objective of this proposal was to evaluate the treatment with the hydroalcoholic extract of the medicinal species *A. citriodora* Palau on depressive-like behaviors in rats subjected to chronic stress in childhood and on mechanisms involved in neuroprotection through the evaluation of neuroinflammation and oxidative stress. The stress caused by the maternal deprivation (MD) protocol in rodents culminated in a significant increase in depressive-like behaviors in adulthood, an increase in the expression of inflammatory substances IL-1 β and IL-6 in the animals' hippocampus, and an increase in the activity of oxidative stress parameters, such as myeloperoxidase (MPO) and TBARS, in serum and hippocampus. The treatment with *A. citriodora* reversed depressive-like behaviors similar to the group treated with escitalopram. Regarding markers of inflammation in nervous tissue, it was observed that animals treated with *A. citriodora* had lower levels than those treated with escitalopram and the control group, and even more significantly when compared to the MD group treated with saline. The treatments reduced MPO activity in the serum of animals that underwent the MD protocol. TBARS was decreased in the serum and ultimately reversed in the hippocampus.

Introduction

Major depressive disorder (MDD) is a debilitating mental condition that affects millions of people around the world [1], characterized by a persistent feeling of sadness or loss of interest in previously pleasurable activities, resulting in significant impairment in the individual's functioning [2]. Although it is often treatable with psychotherapy and antidepressant medication, there are cases in which patients do not respond adequately to conventional treatments, characterizing treatment-resistant depression (TRD) [3, 4].

Adverse childhood experiences significantly impact mental health, potentially increasing the risk of diagnosis or severity of MDD as well as TRD [2]. Maternal deprivation (MD) in rodents simulates early life stress in humans. This method involves separating puppies from their mothers for a specific period during the early development phase. Such separation can induce behavioral and neurochemical responses that resemble the effects of early stress in humans, such as mood dysregulation, anhedonia, changes in sleep patterns, increased anxiety, and reduced ability to cope with stress [5]. Changes in oxidative stress and inflammation parameters are identified in studies with MDD in humans and animals with depressive-like behaviors [5, 6] and are related to its severity and TRD [7].

Oxidative stress, an imbalance between the production of reactive oxygen species and the body's antioxidant capacity, is crucial in modifying biological molecules, activating different transcription factors, and increasing anti- and pro-inflammatory cytokines [8]. These modifications significantly impact the cellular environment, leading to a rise in pro-inflammatory cytokines exerting several bodily functions, including regulating pathways associated with MDD. These pathways range from neurotransmitter metabolism to neuroendocrine functions and neural plasticity [9]. In patients with depression, it is expected to observe elevated levels of pro-inflammatory mediators, such as interleukins (IL)-1, IL-2, and IL-6, and tumor necrosis factor- α (TNF- α) [10]. Furthermore, through activating inflammatory cells, patients with MDD present an increase in oxidative stress markers [11]. Childhood stress and MD alter the oxidative balance and inflammatory parameters [12, 13], especially in serum and limbic brain tissues such as the hippocampus [14, 15].

Regarding the registration of pharmacological compounds already consolidated in therapy as antidepressants, escitalopram is a classic antidepressant belonging to the group of selective serotonin reuptake inhibitors (SSRIs). Among the most studied biological mechanisms currently are the positive function in the intracellular protein complex called mammalian target of rapamycin (mTOR) [16], brain-derived neurotrophic factor (BDNF) [17]), oxidative balance [18] and immune function [19]. Although, like all existing pharmacological therapies, escitalopram is also ineffective for many patients. Studies show that escitalopram acts on neurophysiological mechanisms, exerting neuroprotective effects. Therefore, escitalopram is an essential positive control in research into the possible antidepressant effect of bioactive compounds from medicinal plants.

The pathophysiological mechanisms of MDD can be treated with the use of medicinal plants [20], such as *Aloysia citriodora* (*A. citriodora*), which is associated with anxiolytic, antidepressant effects, antioxidant function, and neuroprotective effects [21]. Studies on the behavioral and biological activities of the medicinal species, mainly in animals and in vitro protocols, provide evidence that *A. citriodora* promotes sedative, anxiolytic, antidepressant, anesthetic, antioxidant, and neuroprotective effects, in addition to antimicrobial and antineoplastic effects [22, 23].

The aqueous extract of *A. citriodora* demonstrated soothing effects in the open-field test carried out with rats, and its action was enhanced with diazepam. The sedative actions were sensitive to antagonism by flumazenil, suggesting that the biological activity of *A. citriodora* occurs through central receptors of the GABAergic system. In α -adrenergic receptors, the extract non-competitively blocked the action of phenylephrine in the vas deferens, and in the heart, it induced negative inotropism. Benzodiazepine-type sedation, negative inotropism, and antispasmodic effect pre-clinically justify its widespread use in abdominal cramps and as an adjunct in the treatment of anxiety [24].

The species presents artemisinin and hesperidin, highly anti-inflammatory and antioxidant substances strongly related to antidepressant properties [25]. Studies in animal models have confirmed the antidepressant effect of *A. citriodora* through different extracts, such as ethanolic, chloroform, and aqueous [26]. Furthermore, research has indicated the antidepressant potential of other species of *Aloysia* [27].

Considering the need to explore the biological mechanisms involved in depression, the need for new therapeutic strategies, and the fact that studies on medicinal species suggest a therapeutic potential of the species, this study evaluated the effect of treatment with the hydroalcoholic extract of *A. citriodora* (HEAc) on depressive-like behaviors, oxidative balance, and inflammatory and neuroprotective mechanisms in rats subjected to MD stress in the first days of life.

Methodology

To carry out this study, analytical, quantitative research of an experimental nature was used. The preparation of plant material, production of extracts, fractions, and isolation of chemical substances using chromatographic methods was carried out in the Pharmacognosy Laboratory at the Community University of Chapecó Region (UNOCHAPECÓ) after approval by the Ethics Committee on Animal Use (CEUA) from UNOCHAPECÓ under protocol n° 005/CEUA/2021.

The animal experiment was conducted at the Bioterism Center (CBI) of UNOCHAPECÓ, a facility registered with the Regional Council of Veterinary Medicine of Santa Catarina (CRMV/SC) n. SC-08771-PJ. Under the supervision of the responsible veterinarian, Natacha Trevisani (CRMV/SC-n°4815SC), this center strictly adheres to the guidelines of the National Council for the Control of Animal Experimentation (CONCEA), ensuring the highest ethical standards in this research.

Brain tissue analyses were carried out in the laboratories of the Federal University of Fronteira Sul, Campus Chapecó, SC.

Collection, botanical identification, and preparation of plant materials

The collection of *A. citriodora* was carried out in Chapecó (SC) (27° 05' 37" S and 52° 39' 58" W) from October to November 2021. The plant material was reduced to tiny fragments and submitted to drying at room temperature ($\pm 25^\circ\text{C}$), protected from direct light and humidity. Next, the dehydrated vegetable drug was crushed in a knife mill (Ciemlab®, CE430), selected in 425 μm sieves (35 Tyler/Mesh), identified and stored away from light.

Hydroalcoholic extract preparation

HEAc was produced by maceration (3 days) with dried plant material (400 g), using ethanol as solvent (70% v/v; 3 l) [28, 29]. After Büchner filtration, the extract was evaporated under reduced pressure (40°C), freeze-dried, weighed (149.485 g; 37.37%), and stored in a freezer at -20°C.

Chemical analysis of the HE Ac by mass spectroscopy

Aiming at the chemical identification of the significant HEAc compounds, the sample (0.010 g) was directly infused into a Thermo Scientific LTQ XL Linear Ion Trap Mass Spectrometer analyzer, equipped with electrospray ionization (ESI) source, in negative mode, rate of drying gas flow 3.0 l/min, drying gas temperature 200°C, spray voltage - 0.5 kV, capillary voltage - 4.5 kV, ECD lens - 10 V, sample flow of 5 $\mu\text{l/h}$ and nebulizer gas flow rate of 0.4 bar.

The samples' fragments (MS/MS) were performed using the collision-induced decomposition (CID) method against argon for ion activation. The first event was a complete mass spectrum scan to obtain data on ions in the range m/z 154–2000. The second scan event was an MS/MS experiment using a data-dependent scan on [MH] molecules of the compounds of interest at a collision gas flow rate of 30%.

Acquisition of the escitalopram oxalate compound

Escitalopram oxalate was purchased 1000 grams from Laborsan (Company Lepuge, São Paulo, Brazil). The compound has a purity of > 95% and is presented in solid form, powder form, and 1000 grams.

Maternal Deprivation Protocol

Male Wistar rats were deprived of their mother for three hours a day for the first ten days after birth. The MD consisted of removing the puppies from their mother's cage. Non-deprived animals (controls) remained undisturbed in the original cage with their mother. The animals were weaned only on the 21st day after birth. Two experimental groups were formed: the MD Group (divided into three subgroups) and the No Stress Control Group. After reaching 60 days of age, the rats were separated into subgroups (N = 10) and subjected to chronic treatment for 14 days with the substances chosen for the experiment: escitalopram as a positive control at a dose of 10 mg/kg, HEAc at a dose of 30 mg/kg. Control groups for treatments received saline solution. The doses of escitalopram are by works published in the scientific literature [30]. The experimental design is illustrated in Fig. 1. The administration of extracts and drugs was carried out orally using the gavage method. The following experimental groups were designed, considering stress, treatment with a dose of escitalopram and *A. citriodora* extract: Control + Saline; MD + Saline; MD + escitalopram; MD + *A. citriodora*.

Behavioral tests

All behavioral tests were conducted during a single period of the day, morning (08:00–12:00), as defined at the beginning of the project, and started 60 minutes after each treatment. All behavioral tests were performed by an observer blind to the experimental groups. Depressive behavior was assessed using the forced swimming test, as described by Porsolt et al. (1977) (n = 10/group), and exploratory motor activity was assessed using the open field test, as described by Réus et al. (2013) (n = 10/group). Animals subjected to oxidative stress parameters and inflammatory markers were euthanized by decapitation, and blood collection was performed. After brain extraction, the hippocampus was separated based on the histological description of Paxinos and Watson (1986).

Neuro-Immune and Inflammatory Parameters

IL-1 β and IL-6 levels were analyzed in the hippocampus using ELISA kits following the manufacturer's protocols. For hippocampal digestion, 1 ml of 50 mM TRIS HCL was added to each Eppendorf with the hippocampal sample. The eppendorfs were vortexed and kept for 2h and 30 min at room temperature. Then, centrifugation was performed at 3500 rpm for 15 minutes, and the supernatant was collected for analysis. The Peterson method (modified Lowry) was used to quantify proteins. The curve and samples were analyzed in duplicate [31]. In sandwich format, IL-1 β and IL-6 levels were assessed using ELISA kits from Sigma-Aldrich, Brazil. 100 μ l of each standard and sample was added to the appropriate wells, covered the wells, and incubated overnight at four °C with gentle shaking. Then, the solution was discarded, and the plate was washed four times with 1X washing solution (300 μ l per well). The plate was inverted and dried against clean paper towels. 100 μ l of 1x prepared detection antibody was added to each well. The wells were covered and incubated for 1 hour at room temperature with gentle shaking. The solution was then discarded, and the procedure was repeated. 100 μ l of streptavidin solution was added to each well, covered, and incubated for 45 minutes at room temperature with gentle shaking. Again, the solution was discarded, and washing was repeated. 100 μ l of TMB One-Step Substrate Reagent (Item H) was added to each well, which was covered and incubated for 30 minutes at room temperature in the dark, with gentle shaking. 50 μ l of stop solution (item I) was added to each well, and the plate was read at the absorbance of 450 nm immediately. The results were calculated considering the interpolation of the absorbance curve equation by concentration and expressed in picograms per milligram (pg/mg) of protein.

Myeloperoxidase Activity - MPO

Myeloperoxidase (MPO) is a heme enzyme produced by inflammatory mediators and released by leukocytes at the injury site, reflecting the activation of neutrophils and lymphocytes. It is responsible for catalyzing the reaction of chloride ions with H₂O₂. MPO also catalyzes the oxidative coupling of phenol and 4-aminoantipyrine (AAP), forming quinoneimine with a maximum absorbance of 492 nm [32]). MPO activity was assessed using a modified peroxidase system, in which the serum and hippocampus samples were mixed with AAP and H₂O₂, incubated at 37 °C for 30 minutes, and measured spectrophotometrically. The results were expressed as μ M of quinoneimine per mg of protein produced in 30 minutes (μ Mq/mg/30min).

Lipid peroxidation analysis - TBARS

Lipoperoxidations are rapid reactions resulting from the breakdown of polyunsaturated fatty acids, generally quantified using thiobarbituric acid reactive species (TBARS), with malondialdehyde (MDA) being its main component, detected mainly by the reaction with thiobarbituric acid (TBA) [33]. To measure MDA, serum samples were mixed with 55 μ L distilled water, 100 μ L orthophosphoric acid (0.2 M), and 25 μ L TBA (0.1 M). After incubation at 37°C, a spectrophotometric reading was performed to detect the pink product formed, with absorption measured at 532 nm. The results were expressed as nM MDA/mL.

Statistical analysis

Analyses were performed using Statistica 7.0 software, and data were represented as means $\pm$ standard error of the mean. Normality was assessed according to the Kolgomorov-Smirnov test, and differences between means were determined using Analysis of Variance (ANOVA, one-way), followed by Tukey's post hoc test. Values of p < 0.05 were considered statistically significant.

Results

Chemical analysis

The mass spectrum (EM-ESI) in the negative mode of HEAc provided ten polyphenolic compounds (Table 1 and Fig. 2). Among them are organic acids and free and glycosylated flavonoids, which are characteristic of *A. citriodora*.

Table 1
Polyphenolic compounds identified in the mass spectrometry (ESI-IT-MS/MS) of *A. citriodora* (EHA) hydroalcoholic extract

Compostos	[M-H] ⁻	MS ₂	Referências
Quinic acid (1)	191	173, 127, 111, 93, 85	(Vidal-Gutiérrez et al. 2020)
Baicalein (2)	269	251, 241, 225	(Han et al. 2007)
Diosmetin (3)	299	284, 256, 227	(Justesen 2001)
Caffeoyl-glucoside (4)	341	179, 119	(Bastos et al. 2007)
Caffeoylquinic acid (5)	353	191, 179, 173, 135	(Bastos et al. 2007)
Kaempferol-3-O-glucoside (6)	447	285, 257, 151	(Vidal-Gutiérrez et al. 2020)
Quercetin-3-O-glucoside (7)	443	301, 300, 271, 161	(Goufo et al. 2020)
Quercetin-3-O-glucuronide (8)	477	301, 283, 179, 151	(Goufo et al. 2020)
Raffinose (9)	503	341, 323, 263, 221, 179, 161, 143	(Lijina and Gnanesh Kumar 2023)
Dicaffeoylquinic acid (10)	515	353, 191, 179, 173	(Dugo et al. 2009)

Effects of MD and treatments on the forced swimming test

The effects of MD and treatments with HEAc (30 mg/kg) and escitalopram (10 mg/kg) on the parameters evaluated in the forced swimming test are illustrated in Fig. 3. In the forced swimming protocol, the One-way ANOVA showed a significant interaction in immobility time between the non-stress and maternally deprived groups and between treatments ($F = 5.03377$; $P = 0.004727$). Tukey's post hoc test showed that MD significantly increased immobility time ($p < 0.02$) in the forced swimming test. Treatments with *A. citriodora* and escitalopram reversed the MD effect ($p < 0.02$).

Regarding swimming time, a significant interaction between groups was observed in the ANOVA ($F = 5.1787$; $P = 0.005471$). Tukey's post hoc test showed that the MD group had shorter swimming time compared to the group treated with escitalopram ($p < 0.02$), saline control ($p < 0.02$), and the group treated with *A. citriodora* ($p < 0.04$). Finally, there was no statistically significant difference between the groups in the forced swimming climbing test.

Effects of MD and treatments in the open field test

The effects of MD and treatments with HEAc (30 mg/kg) and escitalopram (10 mg/kg) on the parameters evaluated in the open field test are illustrated in Fig. 4. In the open field test, One-way ANOVA analysis revealed no significant interaction between the non-stress and MD groups. Both MD and treatments did not induce significant changes in locomotor activity, assessed through the number of crossings ($F = 2.40$; $p > 0.05$) and rearing ($F = 2.11$; $p > 0.05$) in the open field test.

Levels of IL-1 β and IL-6 in the Hippocampus

The effects of MD, as well as treatment with *A. citriodora* (30 mg/kg) and escitalopram (10 mg/kg) on the levels of the inflammatory cytokines IL-1 β and IL-6 in the hippocampus, are illustrated in Fig. 5. The one-way ANOVA revealed a significant interaction of IL-1 β ($F = 14.77$ and $p < 0.001$) and IL-6 ($F = 12.23$ and $p < 0.001$) between groups. Tukey's post hoc test showed that MD significantly increased IL-1 β levels ($p < 0.05$). *A. citriodora* ($p < 0.001$) and escitalopram ($p < 0.01$) reversed the elevated levels of IL-1 β induced by MD. MD also increased IL-6 levels even more significantly ($p < 0.01$). Treatments with *A. citriodora* ($p < 0.001$) and escitalopram ($p < 0.01$) reversed the elevated levels of IL-6 induced by MD.

MPO and TBARS Activity in Serum and Hippocampus

The effects of MD, as well as treatment with HEAc (30 mg/kg) and escitalopram (10 mg/kg) on MPO and TBARS levels in the hippocampus and serum, are illustrated, respectively, in images 6 and 7. In the analysis, ANOVA indicated no significant interaction between the groups when evaluating hippocampal MPO levels ($F = 0.4689$; $p > 0.05$). Differently, the ANOVA showed a significant interaction between the maternally deprived groups and the non-stress group and between treatments in both serum MPO values ($F = 29.0810$; $p < 0.0001$), serum TBARS ($F = 4.2379$; $p < 0.05$) and TBARS in the hippocampus ($F = 4.42067$; $p < 0.05$).

Tukey's post hoc test showed that MD did not induce a significant increase in MPO levels ($p > 0.05$) in the hippocampus. However, it showed a statistically significant increase in serum ($p < 0.001$). TBARS increased significantly in the hippocampus and serum ($p < 0.05$). *A. citriodora* ($p < 0.05$) and escitalopram ($p < 0.01$) reversed the elevated MPO levels induced by MD in the serum. *A. citriodora* ($p < 0.05$) and escitalopram ($p < 0.05$) reversed the high levels of TBARS induced by MD in the hippocampus. Despite not showing statistical significance, there was a reduction in serum TBARS levels in the treatment groups (escitalopram - $p = 0.077579$; *A. citriodora* - $p = 0.06023$) compared to the MD Saline group.

Discussion

The Verbenaceae family, with its 98 genera and 2614 species worldwide, is a rich source of botanical diversity. In Brazil, a country blessed with diverse biomes, 47 genera distributed in 407 species have been identified, ranging from perennial herbs to subshrubs, found in tropical and subtropical regions [34]. Among these, the species *A. citriodora* Palau stands out not only for its wide food and ethnopharmacological uses [34–36], but also for its untapped potential in the field of pharmacology, particularly at the central nervous system (CNS) level.

Initially, chemical analysis of HEAc using UV/Vis spectrometry revealed a representative amount of flavonoids. This finding agrees with [37], who determined the presence of these compounds in *A. citriodora* leaves collected in different geographic locations. A recent study identified new flavonoids for the species (jaceosidin, leptin, and netrin); however, all had flavone structures [38]. Flavones are very active in the human body. They can act on the CNS, specifically on the benzodiazepine receptors GABAA [39, 40]. On the enzymes monoamine oxidases (MAOs), MAOA and MAOB [41], consequently revealing anxiolytic and anticonvulsant effects as well as antidepressant and anti-Parkinson's disease activities, as it acts as an MAO inhibitor and naturally increases dopamine levels [42].

The present work studied the effect of HEAc and the classic antidepressant, escitalopram, on depressive-like behaviors in the forced swimming test in adult rats subjected to MD stress in the first days of life. Inflammatory markers in the hippocampus of animals were also evaluated through proinflammatory interleukins (IL-1 β and IL-6). Oxidative stress was assessed through the activity of MPO and TBARS, both in the serum and in the hippocampus of the animals.

According to scientific literature, this experimental model was chosen because the neonatal period is fundamental in neuronal development, mainly because animals depend on their mothers at this stage. Studies show that MD induces changes in behavior and can alter inflammatory markers involved in the pathophysiology of MDD [43, 44].

Experimental studies have found marked changes in the cortex and hippocampus of rodents after neuroinflammation induced with lipopolysaccharides. Induction mimics anxiety-like behavior, exacerbates oxidative stress, and generates inflammation due to high lipid peroxidase that increases the expression of pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α [45].

One of the traditional drugs most used to treat MDD, anxiety, and other psychiatric disorders is escitalopram, which acts as an SSRI, keeping the neurotransmitter in the synaptic cleft for longer. The increase in serotonin in the synaptic cleft can overcome a probable deficit in cell signaling during MDD and thus bring clinical relief to some patients.

Although the synaptic modulation of serotonin is not clarified, escitalopram's ability to increase serotonin levels necessary for effective treatment in MDD was reduced by acute inflammation induced in rodents [46].

In this study, two behavioral tests were carried out to evaluate the impacts of MD and treatments: the forced swimming test and the open field test to evaluate possible sedative effects of the treatments.

According to [47] and [48]), the forced swimming test is widely used in experimental animal research to evaluate depressive-like behaviors. These behaviors can be triggered through animal protocols through chronic stress, similar to what occurs with MD.

The ANOVA revealed that the MD group had this indicator increase in the immobility parameter compared to the control group. It was observed that the saline MD group showed a significant increase in immobility, while escitalopram and *A. citriodora* significantly reduced this parameter. Therefore, it is possible to conclude that the treated groups showed a reduction in depressive-like behaviors and behaved in a similar way to the control group without stress, corroborating the scientific literature about the antidepressant effects of *A. citriodora* and escitalopram [26, 48].

Regarding swimming time, another parameter of the forced swimming test, ANOVA, revealed a statistically significant difference between the swimming time of the MD group compared to the control group. As with immobility, statistical similarity was observed between the control, escitalopram, and *A. citriodora* groups. The results obtained in the swimming time test are in line with those seen in the immobility test, confirming, once again, that the treatments are efficient in reversing depressive-like behaviors.

As can be seen, the results obtained in the forced swimming test in the groups studied showed that the HEAc (30 mg/kg) and escitalopram (10 mg/kg) had similar actions to the control group. Thus, both treatments had the effect of reducing depressive-like behaviors induced by stress in early life. These results corroborate the evidence found in the literature, especially when looking at the escitalopram group, which is widely used in research with animal models and acts both in reducing depressive-like behaviors and in acting on critical biological mechanisms for neuronal protection and reduction of inflammation [16, 17, 19].

Some drugs and plants can induce a significant sedative effect and interfere with mobility behavior in animals. An open field test was carried out to evaluate this possible action of drugs and extracts, which, according to [49], aims to measure general exploratory activity in rodents. It uses quantity and quality measurements through different parameters, such as crossings and rearings. ANOVA revealed no statistical difference between the different study groups, which indicates that both MD and treatments with escitalopram and *A. citriodora* extract did not exert sedative or stimulant effects on the animals.

Concerning markers of hippocampal inflammation, it was observed that MD increased the levels of inflammatory interleukins compared to the control group. When subjected to treatment with escitalopram, the interleukin levels elevated by MD were reverted to levels similar to the control group. The group treated with *A. citriodora* reduced interleukin levels to values even lower than the interleukins in the saline control group, which means that the plant extract had great neuronal anti-inflammatory power. It is worth remembering the strong relationship between neuroinflammation and the development of depressive disorders [44, 50–52].

Despite advances in synthetic pharmacological treatments, only 50% of depression cases are reversible. Furthermore, they cause adverse effects and are often expensive. Therefore, medicinal plants, including *A. citriodora*, have been increasingly explored by researchers to improve response and adherence to treatment [25]. Some plants have antioxidant action, regulate the HPA axis, reduce inflammatory mediators, and regulate endogenous molecules such as norepinephrine, acetylcholine, and dopamine [25].

A study investigated the behavioral effects of HEAc leaves in female rats. It demonstrated that the substances were capable of promoting a motor depressant and anxiolytic effect, while in one of the most used tests to assess depression, the Functioning Assessment Short Test (FAST), it was possible to notice that the animal's mobility was comparable to that observed in animals treated with reference antidepressants such as fluoxetine and imipramine [53].

A meta-analysis involving 35 studies analyzed inflammatory biomarkers before and after antidepressant treatment and showed that lack of response to treatment was related to persistent inflammation. However, the excellent response to treatment for MDD was associated with reducing the levels of inflammatory markers. Thus, the study suggests that maintaining high levels of inflammation may favor resistance to MDD treatment [54].

Different studies have observed that in cases of MDD, there is an imbalance between reactive oxygen species and cellular antioxidant capacity, which generates inflammation, tissue damage, abnormal neural signaling, and even neuronal death [55, 56].

MPO is a heme-type enzyme present in nervous tissue cells and neutrophils. The increase in its expression may be related to greater inflammatory and neurodegenerative activity [57]. In some studies, it was found to be increased both in the serum and in the nervous tissue of people with depressive disorder. It may correlate with a refractory depressive disorder, which corroborates the reduced effectiveness of antidepressant drugs and cases of refractory to pharmacological treatment [58].

This present study found that MPO activity was increased in the serum of animals subjected to the MD protocol. It also showed depressive-like behaviors compared to the control. This finding aligns with other studies [57–59].

In the study by [58], an increase in the MPO gene expression was noticed, which could explain refractory depressive-like conditions. This finding corroborates other findings in the literature that correlate oxidative stress with MDD since oxidative imbalance leads to a pro-inflammatory environment and changes in cellular physiology.

When observing MPO activity in the serum of the treated groups, a reduction in its parameters was noted with the use of both escitalopram (10mg/kg) ($p < 0.01$) and HEAc from *A. citriodora* (30mg/kg). Thus, oxidative stress was significantly reduced in the groups that received the treatment. These findings align with other studies in which pharmacological interventions also reduced the activity of this enzyme [5, 44, 60].

In the [60] study, a trial using *A. citriodora* in rats with depressive-like behavior showed reduced cortical MPO levels. Although the hippocampal samples that evaluated MPO did not present statistically relevant values in this work, the literature reports an increase in MPO in different brain regions in animals subjected to MD protocols [44, 60–62].

Another oxidative stress assessment used in this study was TBARS. TBARS is a laboratory method for measuring the amount of malonaldehyde and species reactive to thiobarbituric acid. These are byproducts of the degradation of oxidized lipids, and TBARS is an indirect marker of oxidative stress [56]. Although it is not a specific type of marker, TBARS is generally elevated in depressive disorders [56].

In this study, TBARS was elevated in the serum ($p < 0.05$) and in the hippocampus, compared to the non-stressed control group, which once again corroborates the role of stress oxidative effect in MDD conditions. According to [3], in their meta-analysis, it was found that in 40 studies, TBARS levels were elevated in MDD.

Regarding treatments, TBARS parameters were reduced in the hippocampus and serum in treatment with escitalopram and *A. citriodora*. This reduction demonstrates that the treatments have a therapeutic effect in improving oxidative balance. The literature demonstrates similar reduction patterns in oxidative stress, considering the TBARS marker and its reduction following treatment [44].

As observed, the treatments in serum did not show statistical significance compared to TBARS. This finding may be associated with other factors, considering that it is a non-specific marker of oxidative stress, as there was an improvement in the MPO pattern in the serum. Although the statistical software did not observe this significance, it was observed that there was a tendency for treatments to reduce TBARS levels in serum.

Finally, the results obtained in this research using the HEAc, are promising. The data obtained from behavioral tests and inflammatory and oxidative stress markers were considered relevant, as they show a reversal of depressive-like behaviors in immobility, swimming, and forced swimming tests. Furthermore, it reduced the inflammatory interleukins IL-1 β and IL-6 levels in the hippocampus. Regarding oxidative stress, it reduced MPO activity in serum and TBARS, mainly in the hippocampus. Although the results are promising, further studies with active compounds isolated from the plant are necessary to elucidate the biological mechanisms and how this anti-inflammatory and antioxidant activity induced by the MD protocol occurs.

Conclusion

The stress caused by the maternal deprivation protocol in rodents culminated in a significant increase in depressive-like behaviors in adulthood, an increase in the expression of inflammatory substances IL-1 β and IL-6 in the hippocampus, and an increase in markers of oxidative stress, both in the hippocampus and serum.

The treatment carried out with the HEAc reversed depressive behaviors in a similar way to the group treated with escitalopram. Regarding markers of inflammation in nervous tissue, animals treated with *A. citriodora* had lower values than those treated with escitalopram and the control group. Regarding oxidative stress, treatments reduced MPO activity in serum. TBARS was reduced in serum and hippocampus but was only statistically relevant in hippocampal tissue.

The results obtained through this research indicate that substances present in the species *A. citriodora* have potential use as antidepressants since they reduce depressive-like behaviors in animals in a similar way to classic antidepressants and act strongly in reducing neuroinflammation and in some parameters of oxidative stress, which are directly related to the pathophysiology of depressive disorders.

In this way, this study meets the World Health Organization's [1] concerns, as it considers mental illness, especially depression, as one of the biggest causes of disability and suicide in the world. Furthermore, it is observed that classic treatments fail to reverse the disease in several situations, as it is a multifactorial disease. Finally, it is necessary to seek therapeutic alternatives, and *A. citriodora* proved to be a promising strategy. This highlights the importance of further research to investigate other biological markers involved in MDD and some active compounds of *A. citriodora*, aiming to analyze which compounds have the most potent action on the markers and in relieving depressive behaviors.

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose

Ethics approval

This study was approved by the Ethics Committee of the Community University of Chapecó Region (UNOCHAPECÓ) under protocol nº 005/CEUA/2021.

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Author Contribution

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by AGB, SJBS, SSA, JM, AOAN, HJI, KMK, LAS, BVS, MEDM, WARJ, LCB, GBS, DM, MDB, ZMI. AGB, SJBS and SSA wrote the first draft of the manuscript. ZMI and AGB made reviewing and editing. All authors read and approved the final manuscript.

Data Availability

Data will be made available at a reasonable request.

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Figures

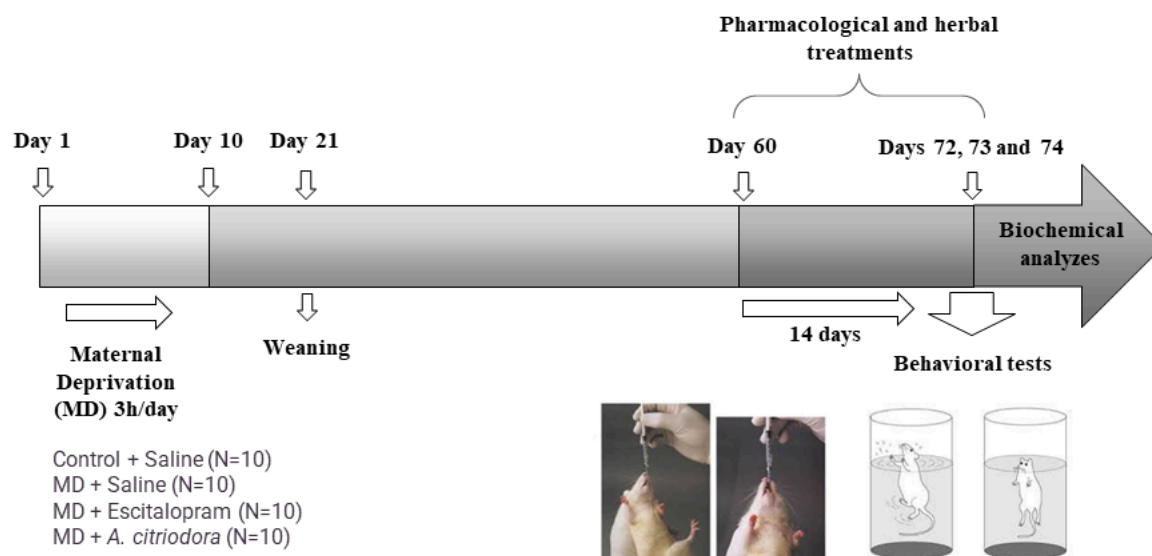


Figure 1

Experimental design of the MD protocol, pharmacological treatments, and behavioral tests.

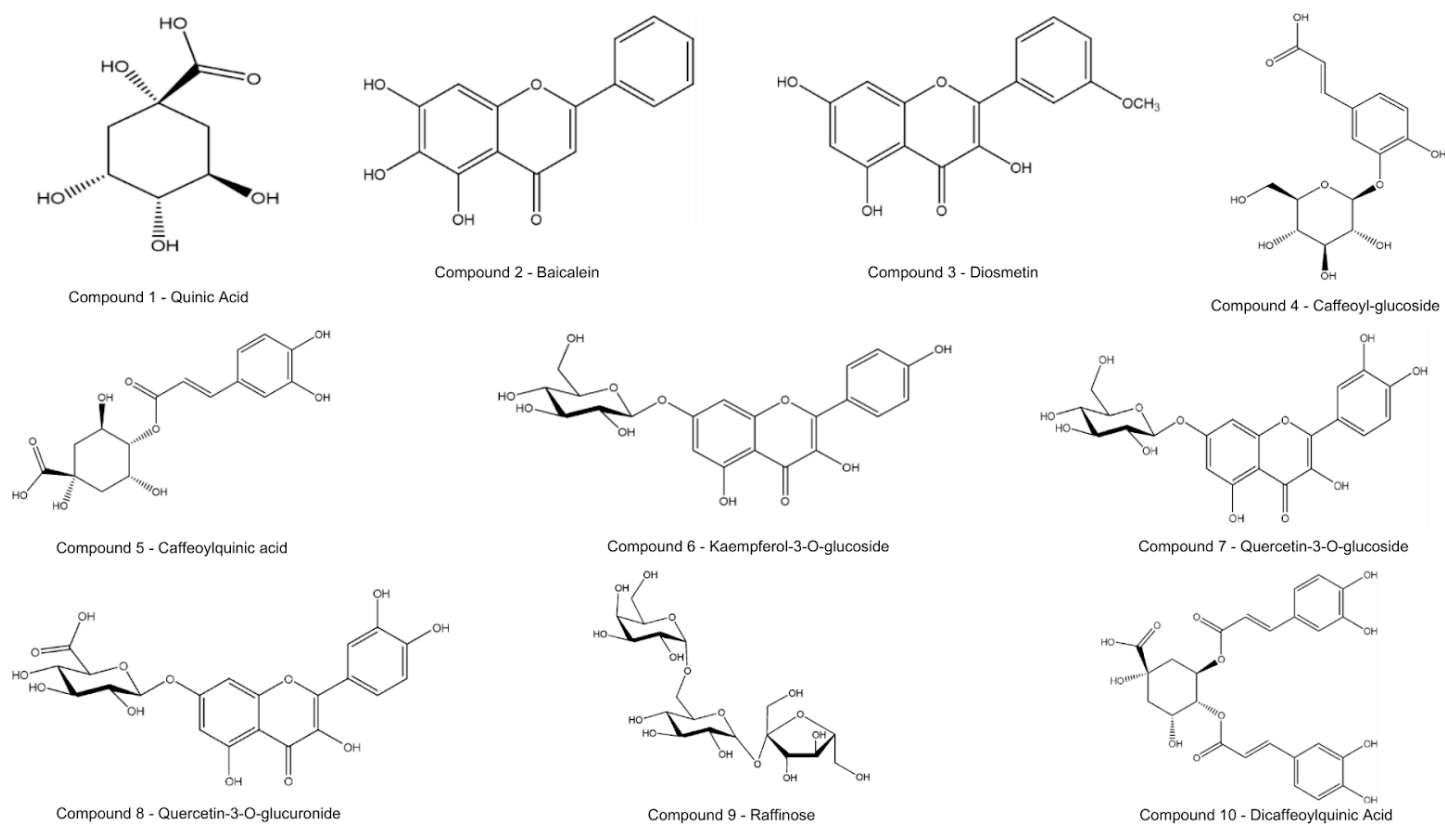


Figure 2

Polyphenolic compounds identified in the mass spectrometry (ESI-IT-MS/MS) of *A. citriodora* (EHA) hydroalcoholic extract.

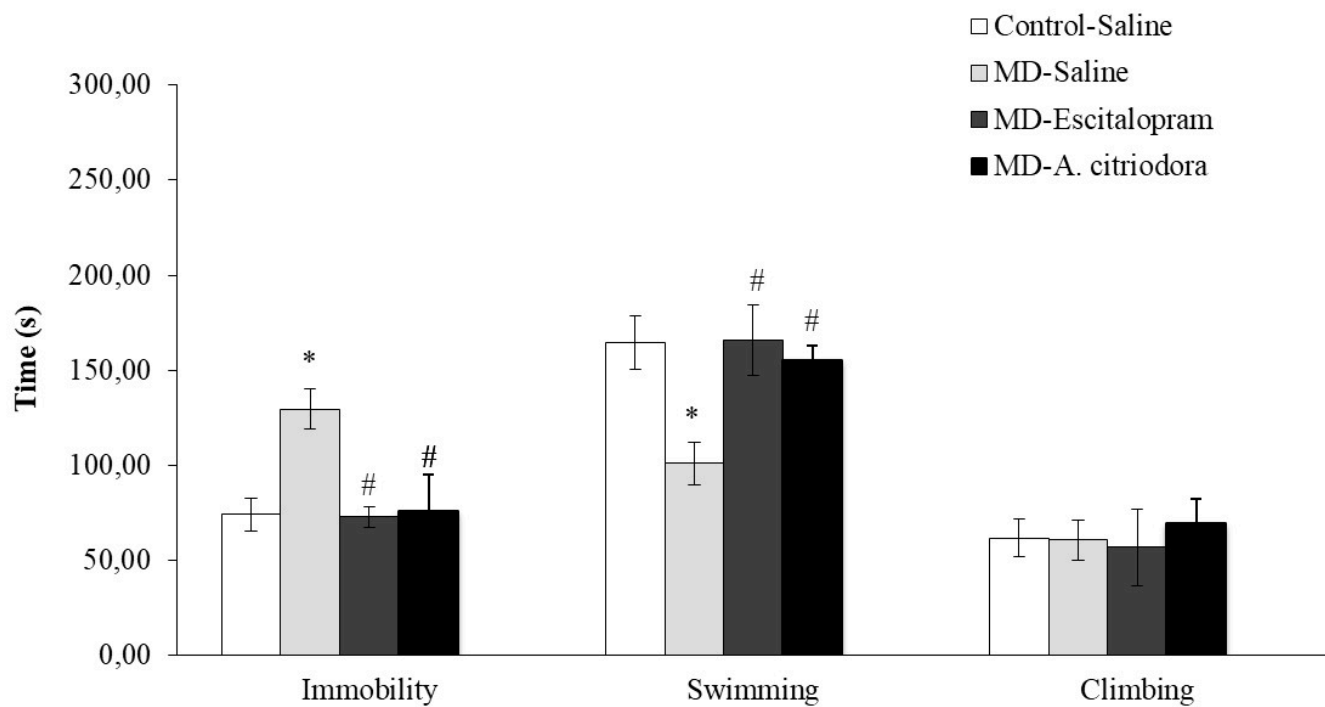


Figure 3

Effects of MD stress and treatments with HEAc (30 mg/kg), escitalopram (10 mg/kg). Data are presented as mean $\pm$ standard error of the mean. Regarding the mobility parameter: *statistical difference between Saline Control and Saline MD ($p < 0.05$); #different from MD Salina ($p < 0.05$). Regarding the swimming parameter: *statistical difference between Saline Control and Saline MD ($p < 0.05$); #different from MD Salina ($p < 0.05$).

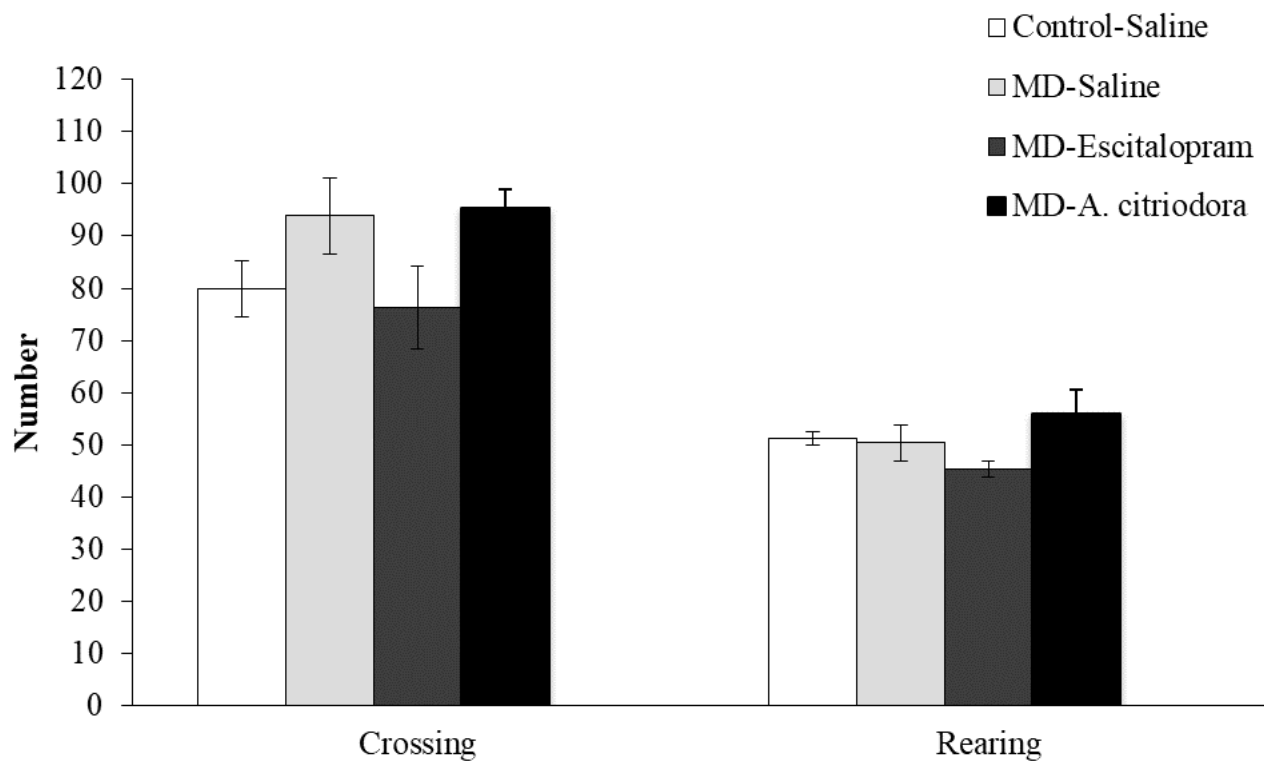


Figure 4

Effects of MD and treatments with HEAc (30 mg/kg) and escitalopram (10 mg/kg) on exploratory motor activity. Data are presented as the mean ± standard error of the mean.

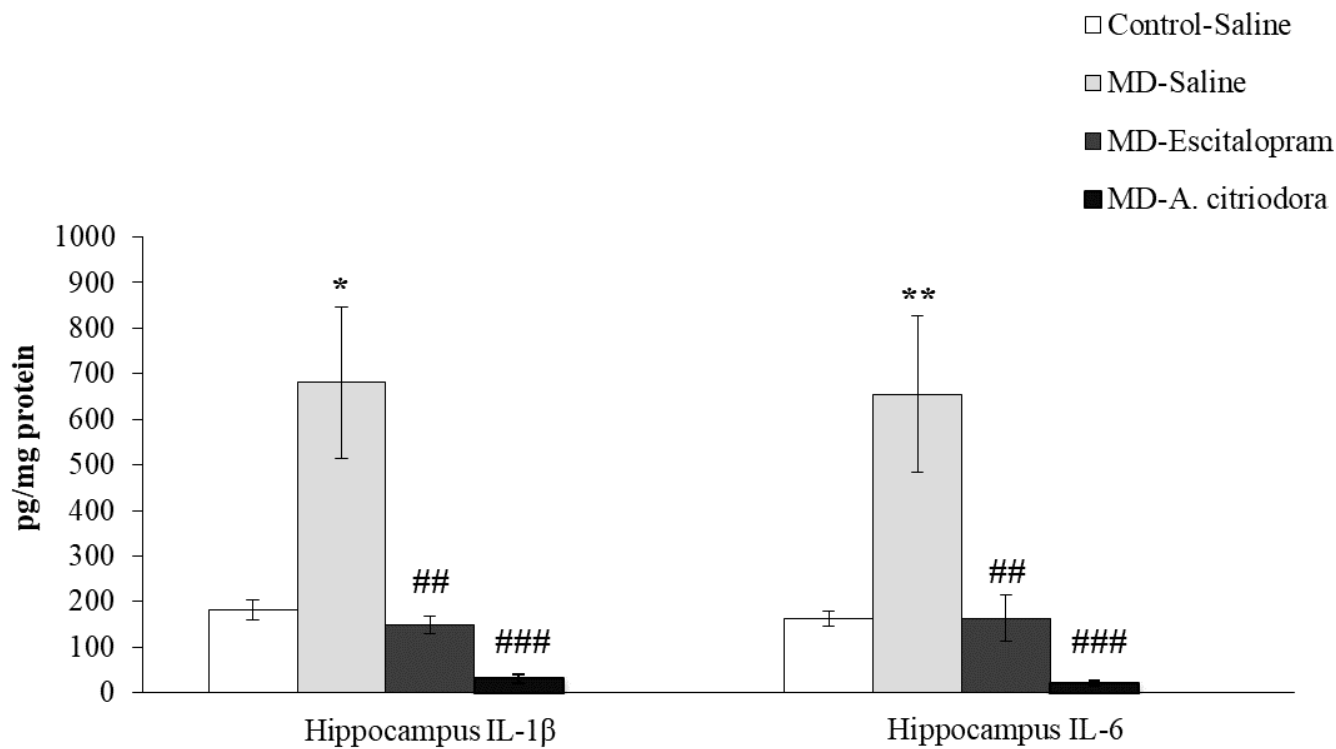


Figure 5

Effects of MD and treatments with HEAc (30 mg/kg) and escitalopram (10 mg/kg) on exploratory motor activity. Data are presented as mean ± standard error of the mean. *statistical difference between Saline Control and Saline MD ($p < 0.05$); **statistical difference between Saline Control and Saline MD ($p < 0.01$); ##different from saline MD ($p < 0.01$); ###different from saline MD ($p < 0.001$).

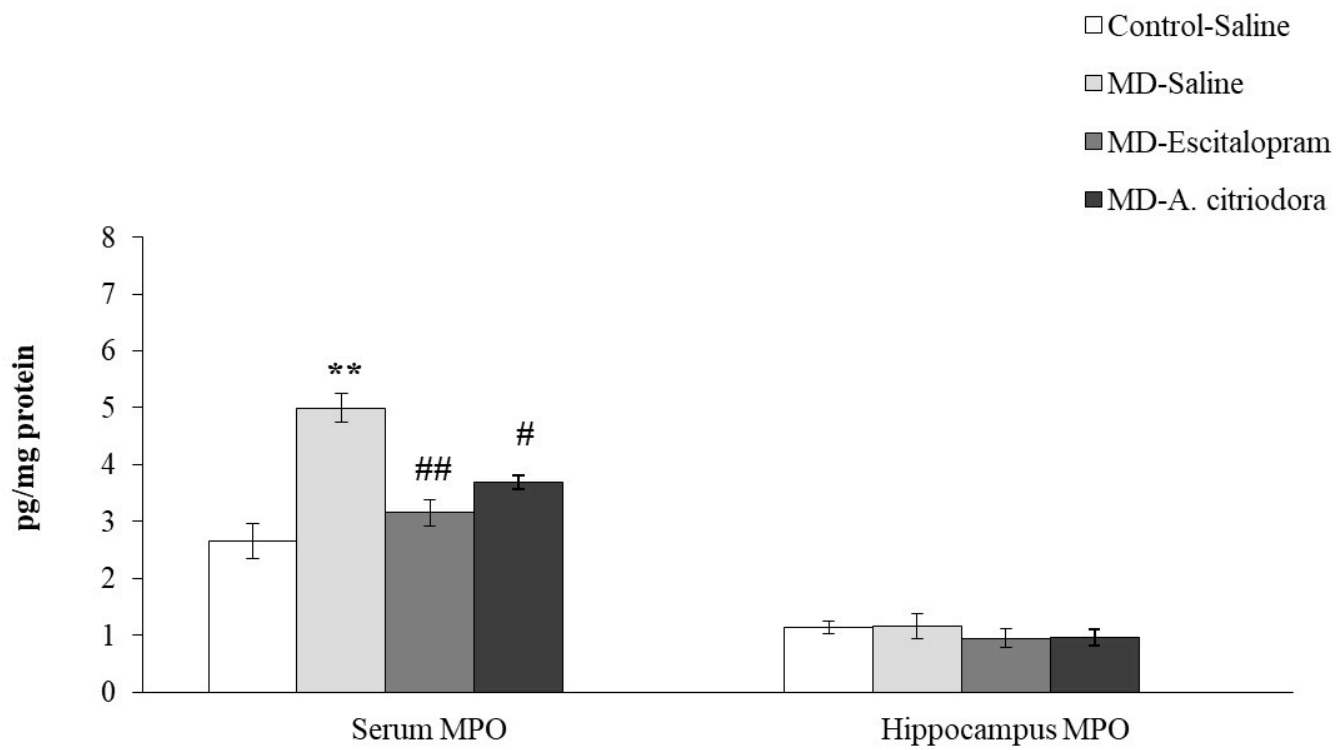


Figure 6

Effects of MD and treatments with hydroalcoholic extract of *A. citriodora*(30 mg/kg) and escitalopram (10 mg/kg) on MPO in serum and hippocampus. Data presented as mean $\pm$ standard error of the mean. #different from MD Salina ($p < 0.001$); *statistical difference between Saline Control and Saline MD ($p < 0.05$); **statistical difference between Saline Control and Saline MD ($p < 0.001$); #different from MD Salina ($p < 0.05$); ##different from MD Salina ($p < 0.01$).

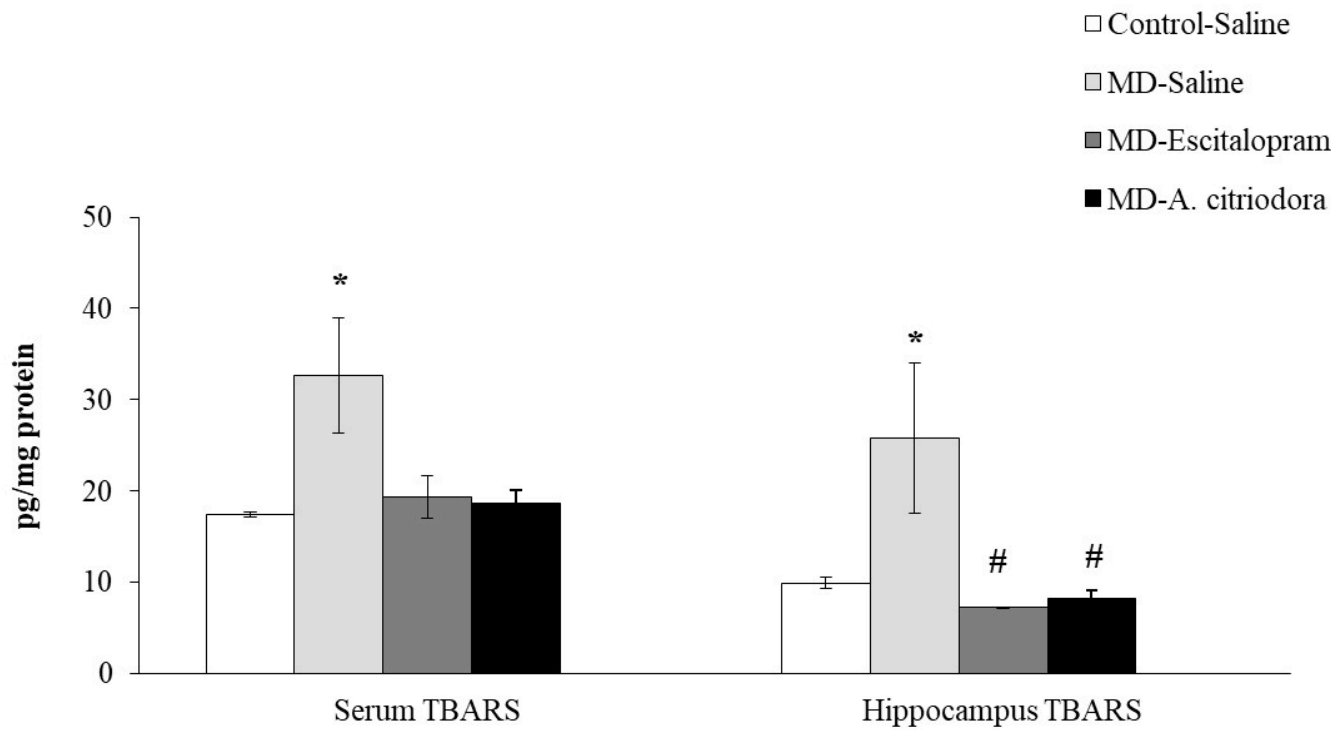


Figure 7

Effects of MD and treatments with hydroalcoholic extract of *A. citriodora*(30 mg/kg) and escitalopram (10 mg/kg) on TBARS in serum and hippocampus. Data presented as mean $\pm$ standard error of the mean. *statistical difference between Saline Control and Saline MD ($p < 0.05$); #different from MD Salina ($p < 0.05$).